A New Method to Evaluate the Unfolding Activity of Chaperone Unit ClpA Based on Fe–S Cluster Disruption

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ATP-dependent proteases unfold their substrates and then refold (*via* chaperone activity) or degrade (*via* protease activity) them. The proteases choose between these two activities by selecting their substrates; however, little is known about their substrate selection mechanism. The present study attempts to clarify this mechanism by investigating the role of the *Escherichia coli (E. coli)* ATP-dependent protease ClpAP. To address this, a reaction system that can measure both chaperone and protease activities simultaneously must be constructed. However, the chaperone activities cannot be evaluated in the presence of protease units. Green fluorescent protein (GFP) is usually used as a model substrate of ClpAP; the fluorescence decrease reflects the degradation of substrates. However, it is difficult to evaluate the chaperone activity of ClpAP using this system, because it cannot distinguish between intact and refolded substrates. Therefore, it is necessary to evaluate the exact unfolding activity while avoiding restoration of substrate spectroscopic characteristics due to chaperone activity. In this study, *E. coli* Ferredoxin (Fd) was used as a new model substrate for ClpAP to evaluate its unfolding activity. Intact and refolded substrates may be distinguished by the existence of an Fd Fe–S cluster. To verify this hypothesis, the absorption spectrum of Fd complexed with ClpA, the chaperone unit of ClpAP, was measured. A decrease in two peaks derived from the Fe–S cluster was observed, indicating that the Fe–S cluster of Fd was disrupted by the ClpA chaperone. This reaction system should prove useful to evaluate the exact unfolding activity of ATP-dependent proteases.

Key word ATP-dependent protease; Fe–S cluster; unfolding activity; ClpA; Ferredoxin

ATP-dependent proteases, such as the eukaryotic 26S proteasome or *Escherichia coli (E. coli)* ClpAP are barrel-like complexes composed of regulatory units and protease units. The former recognize and unfold their substrates, and the latter degrade substrates translocated from regulatory units.[1,2] Besides their unfolding activity, the regulatory units have chaperone activity that remodels the protein structure.[3,4] In other words, ATP-dependent proteases have chaperone and protease activities, and they choose between these activities by selecting the optimal substrates. However, little is known about their substrate selection mechanisms. By elucidating these mechanisms, it will become possible to control the substrate selection of ATP-dependent proteases, and many drugs targeting ATP-dependent proteases may then be developed; for instance, novel antibiotics that increase the degradation activities of ATP-dependent proteases and induce degradation of housekeeping proteins may be developed. In order to clarify substrate selection mechanisms, a reaction system that can measure both chaperone and protease activities simultaneously is needed. However, a method to evaluate the chaperone activity in the presence of a protease unit has not yet been established. ATP-dependent proteases first unfold their substrates,[5,6] and they then remodel (*via* chaperone activity) or degrade (*via* protease activity) them. Therefore, the reaction amount of ATP-dependent proteases can be calculated if the amount of unfolded substrates can be quantified. Hence, it will be possible to quantify the chaperone activity by subtracting the degradation amount from the reaction amount. To construct an evaluation system for substrate selection, we chose *E. coli* ClpAP as a model protease. Green fluorescent protein (GFP) is usually used as a model substrate for ClpAP. GFP in its native conformation emits green fluorescence, and when it is degraded the fluorescence disappears. The protease activity of ClpAP can thus be estimated from the GFP fluorescence intensity.[6] Unfolding of GFP by ClpAP also causes the fluorescence to disappear; however, the fluorescence recovers when the GFP native conformation is restored. Weber-Ban *et al.* evaluated the unfolding activity of ClpA chaperone from the GFP fluorescence intensity by using the GroEL mutant (D87K), which traps the unfolded GFP and prevents it from refolding.[6] However, this method is not suitable to evaluate the substrate selection of ClpAP, because the GroEL mutant in the reaction mixture might affect the substrate selection. Therefore, to evaluate the unfolding activity of the ClpA chaperone and analyze substrate selection, a new model substrate is needed. We decided to use Ferredoxin (Fd) as a model substrate. Fd is an Fe–S protein that has a [2Fe–2S] cluster as part of its native structure. The cluster shows two specific maximum absorbance peaks at nearly 414 and 460 nm.[7] The cluster is disrupted by unfolding of Fd[8] and it does not recover quickly, even when Fd is refolded.[9] Based on these features of Fd, we hypothesize that by using Fd as a ClpAP substrate, the unfolding activity of ClpAP could be evaluated, based upon the amount of Fe–S cluster disruption. In order to prove this hypothesis, we examined whether the ClpA chaperone can disrupt the Fe–S cluster of Fd by measuring the absorption spectrum of Fd in the presence of the ClpA chaperone.

Experimental

Materials All primers for polymerase chain reaction (PCR) amplification were synthesized by Invitrogen Japan K.K. (Tokyo, Japan). The plasmid vector pET15b and the competent cells DH-5α and BL21 (DE3) were obtained from Novagen (Madison, WI, U.S.A.). The restriction endonucleases *Nde*I, *Bam*HI and *Xho*I and Prime STAR HS DNA Polymerase were ob-

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tained from TAKARA Bio Inc. (Shiga, Japan). The ligation mixture was obtained from Nippon Gene Co. (Toyama, Japan). Luria–Bertani (LB) broth was obtained from Sigma Aldrich, Japan K.K. (Tokyo, Japan). Ampicillin sodium and isopropyl-β-thiogalactopyranoside (IPTG) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). ATP and ADP were obtained from Sigma Aldrich, Japan K.K. (Tokyo, Japan). All other chemicals were special reagent grade and were used without further purification.

Plasmids *E. coli clpA* and *fdx* genes were inserted into the *Nde*I and *Bam*HI sites of the N-terminal hexahistidine (His_6)-tag vector pET15b, respectively. The reconstructed plasmids were replicated in *E. coli* strain DH-5α and purified. The *fdx* reverse primer had a ClpA recognition sequence called *ssrA* at its 3′-terminal end. The *Aequorea coerulescens gfp* gene was inserted into the *Bam*HI and *Xho*I sites of the N-terminal glutathione-*S*-transferase (GST)-tag vector pGEX 6p-1 (GE Healthcare). The reconstructed plasmids were replicated in *E. coli* strain DH-5α and purified. The *gfp* reverse primer also had the *ssrA* sequence in its 3′-terminal end.

Expression and Purification of ClpA The recombinant plasmid pET15b-*clpA* was transformed into *E. coli* strain BL21 (DE3) (Invitrogen, Japan). The transformant was picked and used to inoculate 10 ml of Luria–Bertani (LB) medium supplemented with ampicillin (100 μg/ml). Cells were grown at 37 °C overnight. The next day, 5 ml of the culture was used to inoculate 1 l of LB medium containing ampicillin (100 μg/ml). The culture was grown to an A_{600} of 0.7—0.8 and the expression of ClpA was induced by addition of isopropyl β-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. Cells were grown at 28 °C for 2 h and then pelleted by centrifugation (5000 rpm×15 min). The cell pellet (from 250 ml culture) was resuspended in 40 ml of Lysis buffer A (50 mM Tris/HCl (pH 7.4), 2 mM dithiothreitol (DTT), 2 mM ethylenediaminetetraacetic acid (EDTA), 10% Glycerol). Cells were disrupted by sonication (5×30 s), and cell debris was removed by centrifugation at 11000 rpm×90 min. Purification of ClpA was carried out using Ni sepharose resin (Ni Sepharose High Performance; GE Healthcare). The clarified lysate was added to Ni sepharose (equilibrated with Wash buffer A). After that, the resin was washed with 100 ml of Wash buffer A (50 mM Tris/HCl (pH 7.4), 50 mM KCl, 2 mM DTT, 10 mM Imidazole, 10% Glycerol), and then the bound protein was eluted with a linear gradient of Wash buffer A and Elution buffer A (50 mM Tris/HCl (pH 7.4), 50 mM KCl, 2 mM DTT, 250 mM Imidazole, 10% Glycerol). The fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and fractions containing ClpA were dialyzed 3 h×2 with Dialysis buffer (50 mM Tris/HCl (pH 7.4), 50 mM KCl, 10% Glycerol). After that, the ClpA solution was stored at −80 °C.

Expression and Purification of Ferredoxin (Fd) The expression of Fd was carried out as described above. After IPTG induction, the culture was grown at 25 °C for 18 h. The cell pellets from 500 ml of culture were used for purification. The purification was carried out as described above.

Expression and Purification of GFP The expression of GFP was carried out as described above. After IPTG induction, the culture was grown at 25 °C for 4 h. The cell pellets from 250 ml culture were used for purification. The pellets were resuspended with Lysis buffer B (50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1 mM DTT, 2 mM EDTA, 10% Glycerol). Purification of GFP was carried out using Glutathione sepharose resin (Glutathione Sepharose 4 Fast Flow; GE Healthcare). The clarified lysates were added to Glutathione sepharose (equilibrated with Wash buffer B). After that, the resin was washed with 100 ml of Wash buffer B (50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1 mM DTT, 10% Glycerol), and then the bound proteins were eluted with a linear gradient of Wash buffer B and Elution buffer B (50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1 mM DTT, 20 mM reduced Glutathione (GSH), 10% Glycerol). The green fractions were collected and GST-tag cutting protease (PreScission Protease; GE Healthcare) was added. After incubation at 4 °C for 8 h, the reaction mixture was added to Glutathione resin and the flow-through fractions were collected. The green-colored flow-through fractions were stored at 4 °C.

Measurement of Fluorescence Intensity of GFP Fluorescence intensity of GFP was measured on an F-2500 Fluorescence spectrophotometer (Hitachi Co., Tokyo, Japan). The excitation and fluorescent wavelengths were 489 and 508 nm, respectively.

Measurement of Ultraviolet and Visible Absorption Spectrum of Fd UV–visible absorption spectra of Fd were measured on a U3000 Spectrophotometer (Hitachi Co., Tokyo, Japan). The range of measured wavelengths was 200—600 nm.

Quantification of Free Fe^{2+} by the 2,2′-Bipyridyl Method The reaction mixture for 2,2′-bipyridyl method consisted of 300 mM acetate buffer (pH 4.6), 0.125% 2,2′-bipyridyl, and 7.5% ascorbic acid. The reaction mixture of Fd with ClpA was added to the reaction mixture for 2,2′-bipyridyl method. After incubation at 25 °C for 20 min, the absorbance of 522 nm was measured.

Results and Discussion

Evaluation of Unfolding Activity of ClpA from a Decrease in GFP Fluorescence GFP is usually used as a model substrate for evaluating the protease activity of ClpAP.[4] GFP emits green fluorescence when in its native conformation. However, the fluorescence disappears when GFP is degraded by ClpAP. This fluorescence also decreases when GFP is unfolded. Therefore, we explored whether it would be possible to evaluate the unfolding activity of the ClpA chaperone by monitoring decreases in fluorescence, using GFP as a model substrate of the ClpA chaperone. As shown in Fig. 1, the fluorescence of GFP decreased for about 10 min after starting the reaction with the ClpA chaperone in the presence of ATP, then it became constant (Fig. 1A). The amount of fluorescence that disappeared was far smaller than from GFP denatured by addition of 1 mM HCl (Fig. 1B). These results may reflect the fact that GFP was unfolded by the ClpA chaperone, but it was then refolded immediately, so the fluorescence intensity of GFP was apparently constant

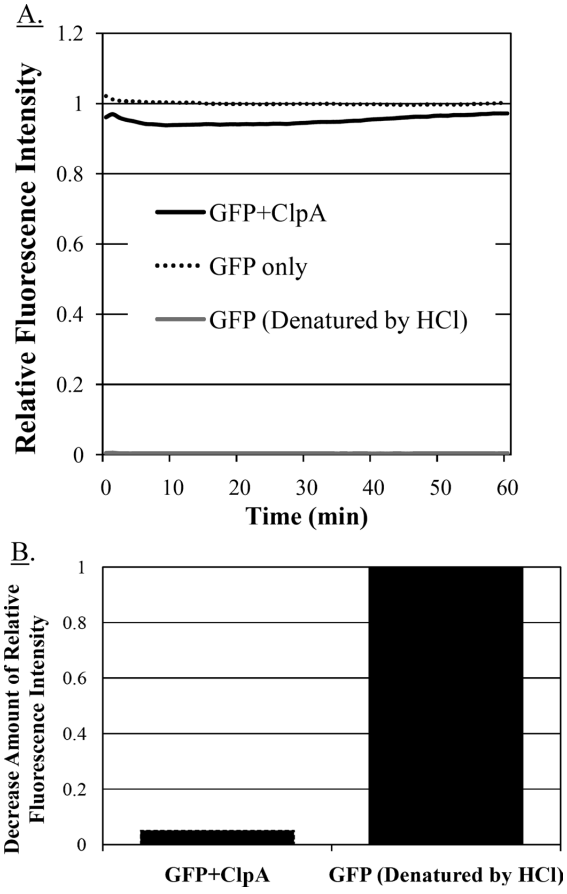

Fig. 1. The Unfolding of GFP by ClpA Chaperone

(A) The fluorescence intensity of 0.5 μM GFP was measured in the presence of 10 mM ATP and in the presence or absence of 2 μM ClpA chaperone (GFP only and GFP+ClpA, respectively) or after addition of 1 mM HCl (GFP (Denatured by HCl)) in 50 mM Tris/HCl (pH 7.4), 300 mM KCl, 30 mM $MgCl_2$, 10% Glycerol at 30 °C for 60 min. The excitation wavelength was 489 nm and the emission wavelength was 508 nm. The fluorescence intensity is the relative intensity when the average fluorescent intensity of "GFP only" is assumed to be 1.0. (B) The decreased amount of relative fluorescence intensity of GFP 10 min after the beginning of the measurement with ClpA chaperone (GFP+ClpA) and GFP denatured by HCl (GFP (Denatured by HCl)). The decreased amounts were calculated by deducting the average relative fluorescence intensity of GFP for 10 min from 1.0.

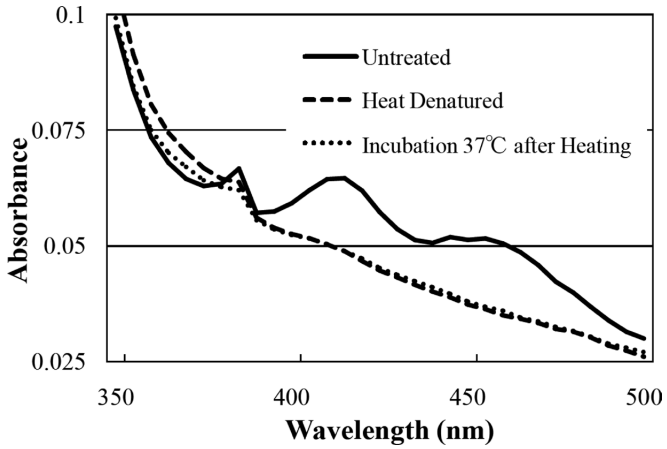

Fig. 2. The Absorption Spectrum Change of Ferredoxin Following Heat-Denaturing

Ferredoxin (10 μM) was heat-denatured by heating it to 60 °C for 30 min, after which the absorption spectrum was measured (Heat Denatured). After that, it was incubated at 37 °C for 1 h and the absorption spectrum was measured (incubation 37 °C after heating).

10 min after the reaction. However, it is difficult to judge the simultaneous reactions (unfolding and refolding) of GFP based on slight changes in the fluorescence intensity. Weber-Ban *et al.* examined whether GFP unfolded by ClpA chaperone could be refolded by addition of a GroEL trap mutant in the reaction mixture.[6] The fluorescence intensity of GFP dropped even further in the presence of the GroEL trap than in its absence. This indicated that GFP was refolded in the absence of the GroEL trap. Therefore, the decrease in the fluorescence intensity of GFP in the presence of ClpA chaperone, as shown in Fig. 1, might reflect the difference between its unfolding and refolding activities. Therefore, we could not evaluate the unfolding activity of the ClpA chaperone using the system with GFP as a model substrate.

Disruption of Fe–S Cluster by Heat Denaturing Ferredoxin (Fd) is an Fe–S protein that has a [2Fe–2S] type Fe–S cluster in its Holo form. It can also exist as a relatively stable Apo form that has no cluster. Therefore, we hypothesized that if the Fe–S cluster of Holo-Fd was disrupted by the unfolding of Fd, it could not be remodeled naturally. To examine this hypothesis, we measured the change in the absorption spectrum after Holo-Fd was heat denatured and incubated at 37 °C after heating. Holo-Fd had two absorption peaks (414, 460 nm, respectively) derived from the Fe–S cluster (Fig. 2). These peaks disappeared after heat denaturing and did not recover after the incubation at 37 °C (Fig. 2). These results suggest that once the Fe–S cluster of Holo-Fd was disrupted and the absorption peaks derived from the cluster disappeared, it did not recover naturally at 37 °C. Therefore, we could evaluate the unfolding activity of the ClpA chaperone by following the disruption of the Fe–S cluster, if the ClpA chaperone could disrupt the Fe–S cluster of Holo-Fd.

Influence of pH on Fe–S Cluster To examine whether pH changes affect the Fe–S cluster formation, we measured the absorption spectrum of Fd under various pH conditions. As shown in Fig. 3, the absorption peaks derived from the Fe–S cluster (414, 460 nm) were observed only at neutral pH (pH 7), but the peaks were not observed at acidic or basic pH

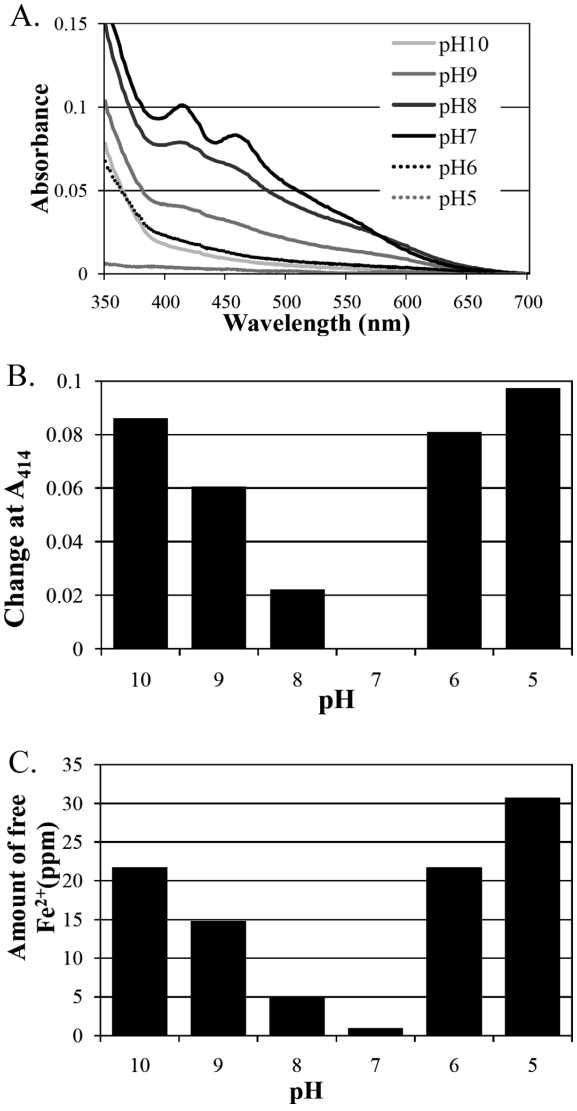

Fig. 3. The Influence of pH on the Fe–S Cluster of Fd

(A) The absorption spectra of Fd under various pH conditions. The acidic and basic pHs were adjusted by adding HCl and NaOH, respectively. The pHs of the reaction mixtures were assayed using pH paper. (B) The change of Absorbance at 414 nm (A_{414}) caused by the pH changes. The decreased amount of A_{414} is calculated by deducting A_{414} at each pH condition from the A_{414} of pH 7 condition. (C) The released amount of free Fe^{2+} caused by the pH changes. The amount of free Fe^{2+} was measured by the 2,2′-bipyridyl method (Experimental).

(pH 5—6 and pH 8—10, respectively) (Figs. 3A, B). The increased amount of free Fe^{2+} in the reaction mixture was also observed at acidic or basic pH (Fig. 3C). These results reflect the fact that the Fe–S cluster of Fd is easily disrupted by a pH change. This confirmed that the pH value is a very important factor in evaluating the exact unfolding activity by ClpA using the Fe–S cluster. When we examine the unfolding activity of ATP-dependent proteases from disruption of the Fe–S cluster, it is important to maintain a neutral pH.

Disruption of Fe–S Cluster by ClpA Chaperone The change in the absorption spectrum of Holo-Fd reacted with the ClpA chaperone and ATP or ADP was measured, in order to examine whether the Fe–S cluster of Holo-Fd was disrupted by unfolding of Holo-Fd by the ClpA chaperone (Figs. 4A, B). As shown in Fig. 4, when Holo-Fd reacted with the ClpA chaperone in the presence of ATP, the absorp-

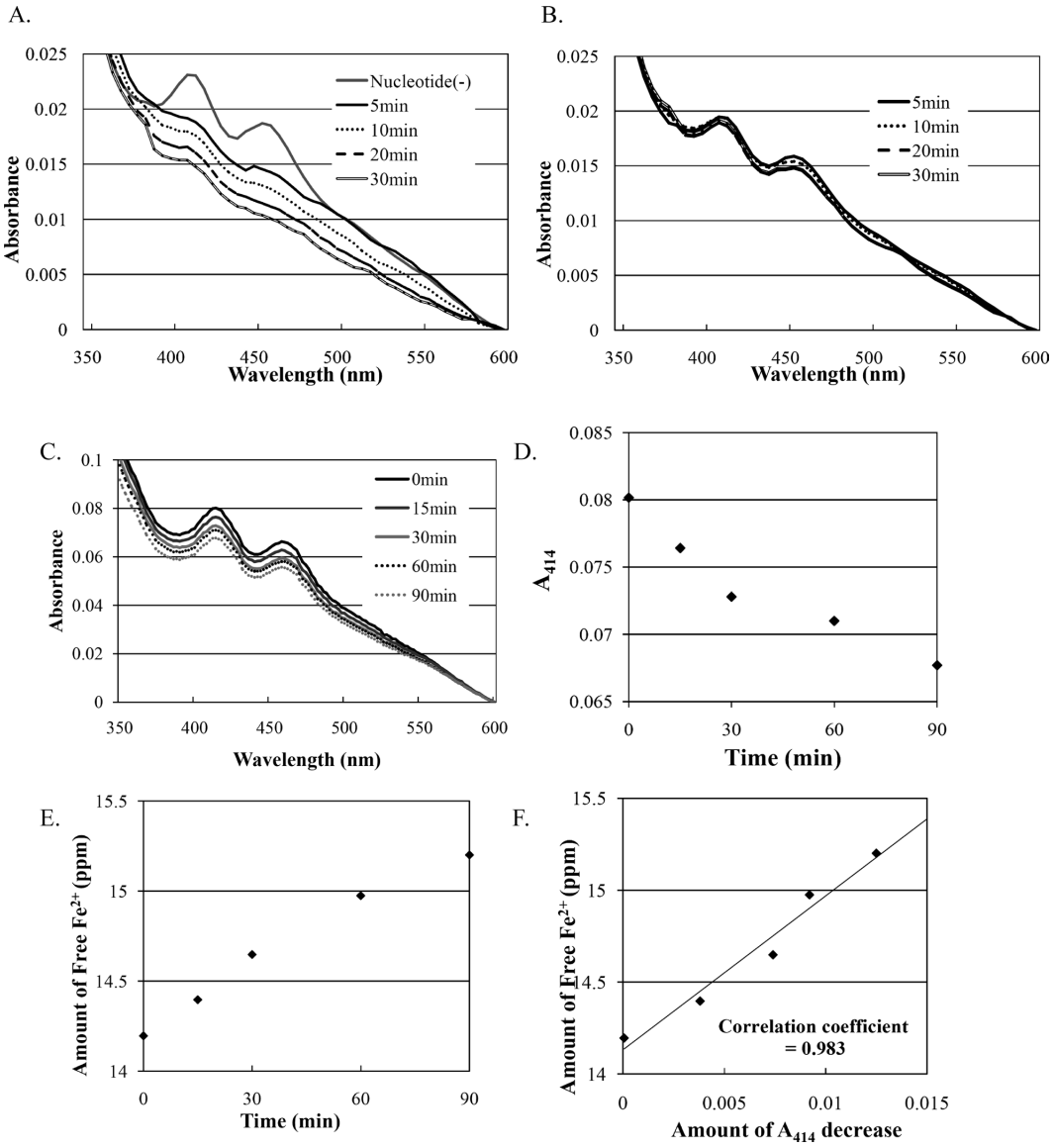

Fig. 4. Disruption of the Fe–S Cluster of Fd by ClpA Chaperone

(A) The absorption spectrum of 10 μM Fd was measured in the presence of 20 mM ATP and 1 μM ClpA at 5, 10, 20, 30 min after reaction in 50 mM Tris/HCl (pH 7.4), 300 mM KCl, 30 mM $MgCl_2$, 10% Glycerol at 37 °C. (B) The absorption spectrum of 10 μM Fd was measured in the presence of 20 mM ADP and 1 μM ClpA at 5, 10, 20, 30 min after reaction in 50 mM Tris/HCl (pH 7.4), 300 mM KCl, 30 mM $MgCl_2$, 10% Glycerol at 37 °C. (C) The absorption spectrum of 50 μM Fd was measured in the presence of 10 mM ATP and 1 μM ClpA at 0, 15, 30, 60, 90 min after reaction in 50 mM Tris/HCl (pH 7.4), 300 mM KCl, 30 mM $MgCl_2$, 10% Glycerol at 37 °C. (D) The change of A_{414} during the reaction of Fd and ClpA. The reaction condition was the same as that shown in Fig. 4C. (E) The changed amount of free Fe^{2+} in the reaction mixture during the reaction of Fd and ClpA. The reaction condition was the same as that shown in Fig. 4C. The amount of free Fe^{2+} was measured by the 2,2′-bipyridyl method. (F) The correlation diagram between the decrease of A_{414} (Fig. 4D) and the released amount of free Fe^{2+} in the reaction mixture (Fig. 4E). The correlation coefficient was 0.983.

tion range 350—600 nm, including the two peaks from Fe–S cluster (414, 460 nm), decreased over time (Fig. 4A). This spectrum change was not seen in the presence of ADP instead of ATP (Fig. 4B). These results reflect the fact that Holo-Fd was unfolded by the ClpA chaperone in the presence of ATP, and the Fe–S cluster was consequently disrupted. We also examined the relationship between the decreased absorption peak derived from the Fe–S cluster (414 nm) and the increase of free Fe^{2+} in the reaction buffer, to clarify whether Fe^{2+} was released from the Fe–S cluster according to the cluster disruption (Figs. 4C—F). During the 90 min reaction, a 15.6% decrease of absorbance at 414 nm (A_{414}) was observed. The amount of free Fe^{2+} increased with the decrease of A_{414}. The correlation coefficient between the change of A_{414} and the increase of free Fe^{2+} in the reaction mixture was very high (0.983). These results indicate that Fe^{2+} was released from the Fe–S cluster after disruption of the Fe–S cluster of Fd by ClpA. Furthermore, the Fe–S cluster could not be remodeled immediately, even if Fd was refolded. Therefore, it is likely that by using Holo-Fd as a model substrate, the unfolding activity of the ClpA chaperone could be evaluated based on the disruption of the Fe–S cluster.

In a system using GFP as a substrate, it is difficult to distinguish between intact and refolded substrates, so the unfolding activity of the ClpA chaperone could not be evaluated in this manner. However, in our system using ferredoxin as a model substrate, it was possible to distinguish between

intact and refolded substrates based on the existence of the Fe–S cluster. In this study, we show that the Fe–S cluster of Holo-Fd was disrupted following unfolding by the ClpA chaperone. This disruption was followed by measuring absorption spectrum changes and quantifying the amount of free Fe^{2+} in the reaction mixture. The results indicated that it would be possible to evaluate the ClpA unfolding activity from the amount of cluster disruption. Thus, the ClpAP unfolding activity could be quantified. As a result, a system could be established to evaluate ATP-dependent protease substrate selection, if the method could be used even in the presence of ClpP.

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